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Anisakid nematode species identification in harbour porpoises (*Phocoena phocoena*) from the North Sea, Baltic Sea and North Atlantic using RFLP analysis

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ABSTRACT

Harbour porpoises (*Phocoena phocoena*) are the only native cetacean species in the German North and Baltic Seas and the final host of *Anisakis* (*A.*) *simplex*, which infects their first and second gastric compartments and may cause chronic ulcerative gastritis. *Anisakis simplex* belongs to the family Anisakidae (Ascaridoidea, Rhabditida) as well as the phocine gastric nematode species *Pseudoterranova* (*P.*) *decipiens* and *Contracaecum* (*C.*) *osculatum*. These nematode species are the main causative agents for the zoonosis anisakidosis. The taxonomy of these genus with life cycles including crustaceans and commercially important fish is complex because of the formation of sibling species. Little is known about anisakid species infecting porpoises in the study area. Mature nematodes and larval stages are often identifiable only by molecular methods due to high morphological and genetic similarity. The restriction fragment length polymorphism (RFLP) method is an alternative to sequencing and was applied to identify anisakid nematodes found in harbour porpoises from the North Sea, Baltic Sea and North Atlantic to species level for the first time. In the study areas, five gastric nematodes from different harbour porpoise hosts were selected to be investigated with restriction enzymes *Hinf*I, *Rsa*I and *Hae*III, which were able to differentiate several anisakid nematode species by characteristic banding patterns. *Anisakis simplex* s. s. was the dominant species found in the North Sea and Baltic porpoises, identified by all three restriction enzymes. Additionally, a hybrid of *A. simplex* s. s. and *A. pegreffii* was determined by *Hinf*I in the North Sea samples. Within the North Atlantic specimens, *A. simplex* s. s., *P. decipiens* s. s. and *Hysterothylacium* (*H.*) *aduncum* were identified by all enzymes. This demonstrates the value of the RFLP method and the chosen restriction enzymes for the species identification of a broad variety of anisakid nematodes affecting the health of marine mammals.

1. Introduction

The harbour porpoise (*Phocoena phocoena*) is a small cetacean species distributed in cold to temperate coastal waters of the northern hemisphere and the only native cetacean in the German parts of the North and Baltic Seas, where they share their habitat with harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) (Read, 1999; Siebert et al., 2001, 2012; Stringell et al., 2015; van Neer et al., 2015). The North Sea and North Atlantic harbour porpoise populations are classified as least concern (LC) on the IUCN Red List of Threatened Species, but especially the Baltic Sea subpopulation is of concern and classified as critically endangered (CR) (Hammond et al., 2008; HELCOM, 2013). Current threats to harbour porpoises in the North and Baltic Seas as well as the North Atlantic are mainly effects of anthropogenic activities like bycatch, overfishing, pollution, habitat loss and underwater noise, but also infectious diseases, most of all parasitic, bacterial and viral infections (Koschinski, 2001; Siebert et al., 2001, 2006, 2012). Harbour porpoises are infected with a variety of ecto- and endoparasites, especially the respiratory and gastrointestinal tracts are affected (Siebert et al., 2001; Lehnert et al., 2005). Porpoises are final hosts of anisakid nematodes, which are gastric endoparasites that infect the first and second stomach compartments of cetaceans and may cause chronic

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ulcerative gastritis (Siebert et al., 2001; Lehnert et al., 2005). These generalist nematodes belonging to the superfamily Ascaridoidea are distributed worldwide (Szostakowska et al., 2002; Strømnes, 2014). They infect a large variety of aquatic animals like marine mammals, fish and cephalopods (Mattiucci et al., 2017). *Anisakis* spp. like *Anisakis simplex* sensu lato (s. l.) with cetaceans as final hosts and *Pseudoterranova decipiens* s. l. and *Contracaecum osculatum* s. l. (all Anisakidae) with pinnipeds as final hosts are the most common species, which can cause the zoonosis anisakidosis, but also *Hysterothylacium aduncum* (Raphidascarididae) with fish as final hosts (Hochberg and Hamer, 2010; Smrzlic et al., 2012; Skrzypczak et al., 2014).

In contrast to many other marine mammal parasites, the life cycle of anisakid nematodes is relatively well understood (Smith and Wootten, 1978; Nagasawa, 1990; Klimpel et al., 2004). It encompasses L3 larvae, which hatch from eggs excreted in faeces by the final hosts. The larvae are ingested by invertebrates, which are preyed on by fish or cephalopods as paratenic hosts. Anisakid nematodes mainly utilise crustaceans such as euphausiids (Smith, 1983), copepods (Klimpel et al., 2004) and amphipods (Klimpel and Rückert, 2005) as intermediate hosts. Infectious L3 larvae are consumed by marine mammal final hosts and mature in their stomach (Aibinu et al., 2019, Centers for Disease Control and Prevention (CDC) 2019).

L3 larvae of *A. simplex* s. l. are located in the body cavity, musculature and various organs (Levsen and Berland, 2012) of the paratenic fish host. *Pseudoterranova decipiens* s. l. larvae occur mainly in the musculature (McClelland, 2002), while *C. osculatum* s. l. larvae predominantly infect the liver (Haarder et al., 2014). *Hysterothylacium aduncum* encapsulates in the intestinal mesenteries and viscera, whereas mature stages are found in the digestive tracts (Shih and Jeng, 2002). Anisakid nematodes are transmitted along the food chain, accumulating in piscivorous fish (Hochberg and Hamer, 2010).

Anisakid nematode taxonomy is intricate and underwent several changes in recent years. *Anisakis simplex* s. l. is a complex of morphologically and genetically highly similar sibling species (D'Amelio et al., 2000): *A. simplex* sensu stricto (s. s.), *A. pegreffii* and *A. simplex* C (*A. berlandi*). Furthermore, the genus contains other species, which are very resemblant and were often only recognised as independent species by genetic studies: *A. typica*, *A. physeteris*, *A. schupakovi*, *A. brevispiculata*, *A. ziphidarum*, *A. nascettii* and *A. paggiae* (Mattiucci et al., 2004, 2009; 2014; Nadler et al., 2005; Mattiucci and Nascetti, 2006). The taxonomic situation of the genus *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* is equally complex due to similar morphologies and sibling species. However, anisakid nematode sibling species frequently inhabit distinct geographical ranges and infect a wide range of host species (Paggi et al., 1991; Nascetti et al., 1993; Lymbery and Cheah, 2007). Anisakid nematodes can be difficult or impossible to differentiate morphologically, especially when sibling species or larval stages are targeted (Mattiucci and Nascetti, 2007; Zhu et al., 2007; Mattiucci et al., 2017). Anisakid nematodes in harbour porpoises from the North and Baltic Seas as well as the North Atlantic have previously been identified morphologically as *A. simplex* or *A. simplex* s. l. (Siebert et al., 2001; Lehnert et al., 2014), but more specific information is lacking. *Pseudoterranova decipiens* s. l. may infect cetaceans like harbour porpoises, because they are sympatric hosts with seals in the eastern North Sea (Herreras et al., 1997), but rarely becomes sexually mature in cetaceans (Lick, 1991). Proper anisakid nematode species identification is essential, therefore, for a better understanding of parasite biodiversity, ecology and host and food web relations (Mattiucci et al., 2017). This is missing for harbour porpoise gastric nematodes in the named areas. Molecular methods have proven useful to complement morphology for an unambiguous discrimination between anisakid nematode species (Nadler et al., 2005). A suitable marker for nematode species identification is the ITS1-5.8S-ITS2 region of the ribosomal DNA (Abollo et al., 2003), because this region features significantly higher nucleotide sequence differences among species than within species (Zhu et al., 1998). The RFLP (restriction fragment length polymorphism) analysis uses restriction enzymes to digest PCR (polymerase chain reaction) products and produces species-specific banding patterns in gel electrophoresis for identification. It has proven to be a quick and precise method to differentiate morphologically similar nematode species and sibling species such as *A. simplex* s. l. (D'Amelio et al., 2000; Umehara et al., 2008a; Fang et al., 2010), but also *P. decipiens* sibling species (Brattey and Davidson, 1996; Brunet et al., 2017) as well as *C. osculatum* sibling species (Kijewska et al., 2002; Szostakowska et al., 2002). Furthermore, the RFLP has advantages such as pictoriality, high specificity, especially regarding sibling species, and good reproducibility, but also challenges like large quantities of DNA necessary for digestion as well as demanding methodological procedures (Naaum and Hanner, 2016; Seesao et al., 2016; Tabit, 2016; van Treuren, 2020). This is the first study to identify anisakid nematodes found in harbour porpoises from the North and Baltic Seas and the North Atlantic to species level with the RFLP method. From the large variety of restriction enzymes, *Hinf*I, *Rsa*I and *Hae*III were shown to differentiate between *A. simplex* s. s., *A. pegreffii*, *P. decipiens* s. s. and *C. osculatum* s. s (Abollo et al., 2003; Umehara et al., 2008a). and were selected to determine the anisakid species infecting the gastrointestinal tract of porpoises in the North and Baltic Seas as well as the North Atlantic.

2. Materials and methods

2.1. Study areas and parasite materials

The study areas were the German and Dutch North Sea, German and Swedish Baltic Sea and Norwegian North Atlantic. Five nematode specimens from different infected harbour porpoises (n = 15, five porpoises per study area) were chosen randomly to identify their anisakid nematode species. The harbour porpoises were examined during necropsies performed according to standardised procedures (Siebert et al., 2001; IJsseldijk et al., 2019) between 1998 and 2018 at the following institutions: the Institute for Terrestrial and Aquatic Wildlife Research (ITAW) in Büsum, Germany, the Faculty of Veterinary Medicine, Division of Pathology, Utrecht University, the Netherlands, the Institute of Marine Research, Tromsø, Norway, and the Swedish Royal Museum of Natural History, Stockholm, Sweden. Additionally, three specimens of each *P. decipiens* s. l. and *C. osculatum* s. l. from North Sea and Baltic harbour and grey seals were analysed for comparison. All nematode specimens were fixed in 70% ethanol.

2.2. PCR amplification

One entire nematode from each sample was taken for DNA isolation, using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). After the DNA was extracted, the entire ITS1-5.8S-ITS2 region was amplified by PCR with the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATC ATT-3′) and NC2 (5′-TTAGTTTCTTCCTCCGCT-3′) (Zhu et al., 1998). PCR reactions were performed with 25 µl Mastermix (MyTaq™ Red Mix, Bioline, London, United Kingdom), 1 µl forward primer, 1 µl reverse primer and 5 µl target DNA, replenished with distilled water to a total reaction volume of 50 µl. The PCR started with an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and elongation at 72 °C for 10 s. Reactions were carried out using a Biometra T Gradient thermocycler (Biometra GmbH, Göttingen, Germany). In order to verify the amplification quality, 10 µl of the PCR products were visualised by ultraviolet light (monitor: Santec Peqlab, Peqlab (VWR), Erlangen, Germany; darkroom: Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany) on SYBR® Safe DNA (Invitrogen (Thermo Fisher Scientific), Waltham, USA) (1.5 µl) stained 1.5% agarose gels (UltraPure™ Agarose, Invitrogen (Thermo Fisher Scientific), Waltham, USA), using a 100-bp DNA ladder (4 µl) (Quick-Load®, New England Biolabs GmbH, Frankfurt am Main, Germany) as molecular marker.

2.3. RFLP

The PCR products were digested using the restriction enzymes *Hinf*I, *Rsa*I and *Hae*III. Digestion reactions had a final volume of 25 µl, consisting of 2 µl distilled water, 2.5 µl CutSmart® buffer, 0.5 µl restriction enzyme (*Hinf*I: size: 5000 units/concentration: 10,000 units/ml; *Rsa*I: size: 1000 units/concentration: 10,000 units/ml; *Hae*III: size: 3000 units/concentration: 10,000 units/ml) (both from New England Biolabs GmbH, Frankfurt am Main, Germany) and 20 µl target PCR product. The digestion was performed for 2 h at 37 °C in a HX Mini block heater (Peqlab (VWR), Erlangen, Germany). The products were analysed via electrophoresis with SYBR® Safe DNA (1.5 µl) stained 2% agarose gels and a 100-bp DNA ladder (8 µl) as molecular marker, using the whole digestion product, a voltage of 60V (10V/cm) and 3 h running time, and visualised by ultraviolet light.

3. Results

Amplification of the ITS1-5.8S-ITS2 region produced an approximately 960 bp long fragment for *A. simplex* and *C. osculatum*, about 1000 bp for *P. decipiens* and 1030 bp for *H. aduncum*. Restriction of the North Sea and Baltic nematode samples with *Hinf*I (Fig. 1 a, d) produced three fragments with about 610 bp, 230 bp and 80 bp characteristic for *A. simplex* s. s. One PCR product from a nematode found in a Dutch harbour porpoise showed a unique, characteristically different banding pattern with 610 bp, 330 bp, 280 bp, 230 bp and 80 bp fragments to all other products (Fig. 1 a, lane 2). *Rsa*I restriction of the North Sea and Baltic porpoise nematodes (Fig. 1 b, e) resulted in approximately 850 bp, 540 bp, 400 bp, 310 bp and 120 bp fragments, of which the 540 bp, 400 bp and 310 bp fragments were the most prominent, characteristic for *A. simplex* s. s. Restriction of the North Sea and Baltic porpoise nematodes with *Hae*III (Fig. 1 c, f) produced approximately 610 bp and 180 bp fragments with an additional 350 bp fragment, a specific pattern of *A. simplex* s. s. Anisakid nematodes sampled from harbour porpoises off Norway were analysed in comparison to the infected North Sea and Baltic porpoises. The banding pattern of *A. simplex* s. s. as described above was found in three cases (Fig. 1 g-i, lane 1, 2, 5), whereas lane 3 and 4 displayed a different RFLP profile. *Hinf*I restriction of the sample in lane 3 resulted in approximately 700 bp and 400 bp fragments, whereas *Rsa*I produced 610 bp and 220 bp fragments and *Hae*III 340 bp, 260 bp, 210 bp, 120 bp and 80 bp fragments, of which the largest three were the most prominent, characteristic for *H. aduncum*. The restriction of the sample in lane 4 with *Hinf*I produced about 700 bp and 180 bp fragments, *Rsa*I generated 280 bp, 250 bp, 180 bp and 100 bp fragments and *Hae*III 580 bp and 320 bp fragments, specific for *P. decipiens* s. s.

*Hinf*I, *Rsa*I and *Hae*III also differentiated between *A. simplex* s. s., *P. decipiens* s. s. and *C. osculatum* s. s., for which *Hinf*I produced approximately 500 bp and 480 bp fragments, *Rsa*I 380 bp, 260 bp and 240 bp fragments and *Hae*III 430 bp, 360 bp and 180 bp fragments, showing characteristic and different banding patterns (Fig. 1 j-l).

4. Discussion

Twelve individual specimens of *A. simplex* s. s., one individual of *H. aduncum* and one individual of *P. decipiens* s. s. were identified in porpoises from the study areas, as well as one hybrid. The results of the North Sea and Baltic RFLPs in this study indicate that *A. simplex* s. s. is the dominant *Anisakis* spp. in harbour porpoises from these habitats. This is supported by previous studies (Mattiucci and Nascetti, 2006; Mattiucci et al., 2017). The only exception is the unique RFLP profile observed in a nematode sampled from a porpoise from the Dutch North Sea displayed in Fig. 1 a, lane 2, which was identified as a hybrid of *A. simplex* s. s. and *A. pegreffii* (Umehara et al., 2008b). *Anisakis pegreffii* is distributed in cetaceans in the Mediterranean Sea and the Atlantic Ocean near Portugal and Spain in European waters (Abollo et al., 2003). Harbour porpoises are a highly mobile and wide-ranging species (Embling et al., 2010), but the population in Iberian waters seems to be genetically isolated from the Northeast Atlantic population ranging from the northern Bay of Biscay to Norway and Iceland (Fontaine et al.,

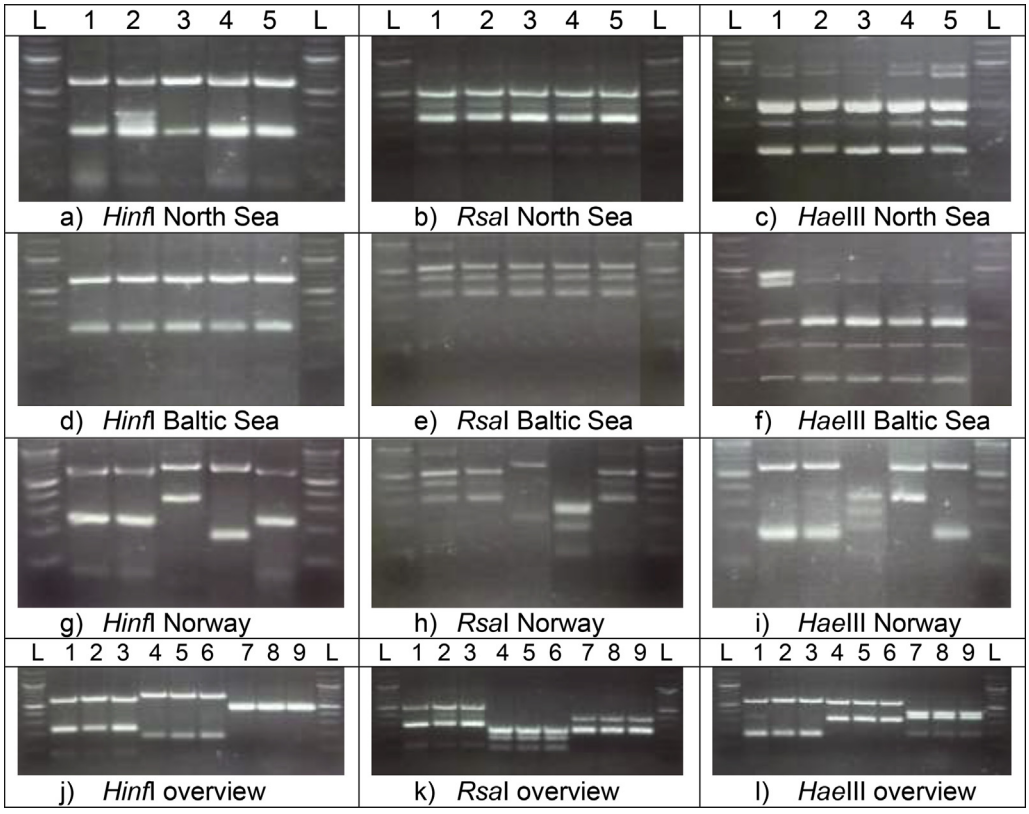

Fig. 1. RFLP profiles obtained by digestion of ITS1-5.8S-ITS2 region with the restriction enzymes *Hinf*I, *Rsa*I and *Hae*III. a)-i) lane 1–5: Anisakid nematodes from harbour porpoises. j)-l) lane 1–3: *A. simplex* s. s. from North Sea, Baltic and Norwegian harbour porpoises; lane 4–6: *P. decipiens* s. s. from North Sea and Baltic harbour and grey seals; lane 7–9: *C. osculatum* s. s. from North Sea and Baltic harbour and grey seals. L: 100-bp ladder.

2007, 2014), similarly to Black Sea and Baltic porpoises (Wiemann et al., 2010; Lah et al., 2016). Therefore, this finding may be due to migrating prey species acting as transport host. The infected porpoise had multiple whiting (*Merlangius merlangus*) otoliths in its stomach (Leopold, pers. comm.), indicating the origin of the hybrid. Whiting and other anisakid nematode paratenic hosts like herring (*Clupea harengus*) and cod (*Gadus morhua*) are part of the diet of North Sea and Baltic harbour porpoises (Gilles, 2009; Leopold, 2015; Andreasen et al., 2017). Herring and cod are common intermediate or paratenic hosts for *A. simplex* s. s. in the North Sea (Klimpel and Palm, 2011; Kuhn et al., 2013). This equally applies to whiting (Pierce et al., 2017), which habitat reaches to the northern coast of the Iberian Peninsula (FAO, 2020). Here, *A. simplex* s. s. and *A. pegreffii* sympatrically occur in fish and cetaceans also as mixed infections (Abollo et al., 2001, 2003). Furthermore, *A. simplex* s. s. and the hybrid of *A. simplex* s. s. and *A. pegreffii* were found in a minke whale (*Balaenoptera acutorostrata*) from the North Pacific Ocean (Umehara et al., 2008b), with resembling banding patterns and fragment lengths as shown in our study. In conclusion, the RFLPs of the North Sea samples showed that it may be important to use a set of restriction enzymes for identification of hybrids, which in this study was only revealed by *Hinf*I.

The situation of anisakid nematode life cycles in the Baltic Sea is different compared to the North Sea. Low salinity in the Baltic Sea is unsuitable for many euphausiid intermediate hosts and may therefore impede *Anisakis* spp. life cycles (Zander and Reimer, 2002). *Anisakis* spp. are mainly transported to the Baltic Sea by migrating herring (Grabda, 1974; Podolska and Horbowy, 2003). However, *A. simplex* s. s. and other anisakid nematode species have been found in invertebrates ingested by Baltic cod, indicating a possible completion of the life cycle (Pawlak et al., 2018, 2019). Here, it is essential to identify the anisakid nematode species properly in order to track their origin and assess their health impact on the threatened Baltic harbour porpoise population.

In the Norwegian samples, *A. simplex* s. s. predominated, although *H. aduncum* and *P. decipiens* s. s. were identified additionally. *Hysterothylacium aduncum* is assumed to have been infecting a prey item as it matures in fish (Herreras et al., 1997). In Norwegian waters, harbour porpoises mostly feed on squid, crustaceans and various fish species like herring, cod, whiting, saithe (*Pollachius virens*) and Mueller's pearlside (*Maurolicus muelleri*) (Bjørge, 2003; Moan, 2016), including multiple paratenic hosts of anisakids (Aspholm et al., 1995; Klimpel et al., 2004; Levsen and Lunestad, 2010). *Pseudoterranova decipiens* s. s., *H. aduncum* as well as *C. osculatum* s. s. infect a resemblant variety of fish host species as *A. simplex* s. s. in the North and Baltic Seas (Klimpel et al., 2003; Lunneryd et al., 2015). Previously, *A. simplex* s. s. was identified by RFLP in false killer whale (*Pseudorca crassidens*) and long-finned pilot whale (*Globicephala melas*) from the Pacific and North Atlantic Oceans (D'Amelio et al., 2000) with similar fragment lengths. Fragment lengths from RFLPs of *A. simplex* s. s. in cetaceans from the families Delphinidae, Kogiidae, Physeteridae and Ziphiidae from the southeast Atlantic coasts of the USA, Gulf of Mexico and Caribbean Sea were similar to this study (Cavallero et al., 2011). *Pseudoterranova decipiens* s. s. typically infects harbour and grey seals, which share habitat and prey with harbour porpoises in Norwegian waters (Aarefjord et al., 1995). Together with *C. osculatum* s. s. it was identified by RFLP in harbour and grey seals from the Canadian Atlantic Ocean (Brattey and Davidson, 1996) and the Baltic Sea (Kijewska et al., 2002). They were found also in ringed seals (*Pusa hispida*) (Pufall et al., 2012) and beluga whales (*Delphinapterus leucas*) from the Canadian Arctic beside *A. simplex* s. s. (Najda et al., 2015), with fragment lengths similar to those in this study. *Pseudoterranova decipiens* s. s. rarely becomes mature in cetaceans, because it is adapted to pinnipeds as final hosts (Aspholm et al., 1995; McClelland, 2002). Larval gastric nematodes of seals have been found in harbour porpoises in sympatric areas like Newfoundland and the eastern North Sea, but porpoises are considered to be accidental hosts (Brattey and Stenson, 1995; Herreras et al., 1997). Nonetheless, all three restriction enzymes detected the accidental infections with *P. decipiens* s. s. and *H. aduncum* beside *A. simplex* s. s., indicating their efficiency in identifying various anisakid nematode species in harbour porpoises.

The high macroscopic similarity of anisakid nematodes, especially as larval stages, host mobility and the zoonotic potential necessitate an effective tool like the RFLP for correct species identification. Nematode infections in fish and decreasing fish health have been associated with recovering marine mammal populations by some authors (Jensen and Idås, 1992; Hauksson, 2011; Buchmann and Mehrdana, 2016). However, environmental and anthropogenic factors contributing to fish immunity such as salinity, temperature, prey availability and pollutant exposure from ammunition dumpsites have to be taken into consideration (Baršienė et al., 2014; Lang et al., 2017, 2018). In complex ecosystems like the Baltic and nematodes with trophic transmission and multi-host life cycles it seems doubtful that there are simple, direct links (Valtonen et al., 2004). Anisakid nematode prevalence in harbour porpoises differs in geographic region, reflecting oceanographic conditions and distribution of prey species (Lehnert et al., 2005, 2014). In the study areas, anisakidosis has been caused rarely, in part by *A. simplex* s. l. (Van Thiel et al., 1960; Eskesen et al., 2001; Kołodziejczyk et al., 2020), but also by *P. decipiens* s. l. (Skirnisson, 2006; Brunet et al., 2017) and *C. osculatum* s. l. (Schaum and Müller, 1967), mostly due to privately prepared raw, salted or marinated fish products. Furthermore, a few human infections with *H. aduncum* were reported worldwide (Yagi et al., 1996; González-Amores et al., 2015). Varying population size and abundance of marine mammals in the study areas as well as complexity and functionality of life cycles have to be considered to evaluate the role of porpoises and seals in the dispersal of anisakid nematodes (Valtonen et al., 2004).

This is the first time harbour porpoise anisakid nematode species have been determined using RFLP. Restriction fragment length polymorphism analysis was proven to be a practical and quick tool for identifying anisakid nematode species, due to its flexibility with various restriction enzymes and high specificity. The RFLP method was able to identify not only *A. simplex* s. s. as the dominant species in the investigated harbour porpoises, but also an anisakid hybrid far from its native habitat. Furthermore, it differentiated four anisakid nematode species with persuasive visual and repeatable means. This study recommends the RFLP as an effective alternative to macroscopic species identification and sequencing of anisakid nematodes in institutions without access to in-house sequencing. Knowledge about anisakid species spectrum in vulnerable marine mammals will help to better understand their diet and habitat preferences as well as evaluate zoonotic risks in coastal environments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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